영상기반 세포수 측정기의 임상적 적용

나위진[1] · 신은경[1] · 고하늬[1,2] · 서진영[2] · 장미수[3] · 남은우[3] · 오종현[4] · 정유미[4] · 이지연[4]
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[1]한양대학교병원 소아청소년과, 한양대학교 의과대학 [2]조혈모이식센터, [3]의학통계지원실, [4]나노엔텍

Clinical Applicability of Newly Developed Image-based Cell Counter for Counting CD34+ Cells: Comparison with Flow Cytometric Analysis

Wee-Jin Rah, M.D.[1], Eun-kyung Shin, M.D.[1], Hani Koh[1,2], Jin Young Suh[2], Misoo Chang[3], Eunwoo Nam, Ph.D.[3], Jong Hyun Oh[4], Yumi Jung[4], Ji-yeon Lee[4], Sung Rok Bong[4], Sung Hun Hong[4], Jee Young Kim[4], Sunmi Han, Ph.D.[4], Jeoung Ku Hwang[4], Chanil Chung, Ph.D.[4] and Young-Ho Lee, M.D., Ph.D.[1,2]

[1]Department of Pediatrics, Hanyang University Hospital, [2]Blood & Marrow Transplantation Center, [3]Biostatistical Consulting and Research Lab, Hanyang University College of Medicine, [4]NanoEntek, Seoul, Korea

Background: Flow cytometric analysis is the standard method for enumerating CD34+ stem cells in hematopoietic stem cell transplantation. However, it has some limitations such as expensive instrumentation, high reagent costs, and discrepancies between technicians and laboratories. We compared counts of total nucleated cells (TNCs) and CD34+ cells counts obtained from a flow cytometer with a newly-developed image-based microscopic cell counter (ADAM II) to evaluate the possibility of clinical application of the ADAM II.

Methods: We used 18 samples of circulating peripheral blood (PB) and waste tube fractions of peripheral blood stem cells (PBSCs) harvested by apheresis after G-CSF mobilization from adult volunteer donors. We assessed the reproducibility and linearity of the new procedure and compared the numbers of TNCs and viable CD34+ cells determined with the ADAM II and two different flow cytometers (FACSCalibur, FACSCanto II).

Results: Numbers of viable CD34+ cells determined with the ADAM II were accurate over the expected range; the intra-assay coefficient of variation was ≤19.8%. Linearity was also satisfactory (R^2=0.99). TNC counts obtained with the ADAM II were highly correlated with those obtained with the FACSCalibur (R^2>0.9841, P<0.0001) and FACSCanto II (R^2>0.9620, P<0.0001), as were the numbers of viable CD34+ cells obtained with the ADAM II and the FACSCalibur and FACSCanto II (R^2>0.9911, P<0.0001 and R^2>0.9791, P<0.0001), respectively.

Conclusion: The newly developed image-based microscopic cell counter (ADAM II) appears to be suitable for enumerating TNCs and viable CD34+ cells.

Key Words: Image-based cell counter, CD34+ cell

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Corresponding Author: Young-Ho Lee
Department of Pediatrics, Hanyang University Hospital, Hanyang University College of Medicine, Hanyang University Hospital, 222-1 Wangsimni-ro, Seongdong-gu, Seoul 04763, Korea
Tel: +82-2-2290-8383
Fax: +82-2-2220-8390
E-mail: cord@hanyang.ac.kr
ORCID ID: orcid.org/0000-0003-1498-2773

Introduction

The number of viable CD34+ cells in peripheral blood (PB) as well as in graft products is the most important parameter for monitoring and predicting clinical outcomes after hematopoietic stem cell transplantation (HSCT) [1-3].

At present, flow cytometry and image-based analysis are the two main techniques used to count fluorescently labeled cells. Although flow cytometric analysis is the standard method for enumerating CD34+ stem cells it has some limitations, such as expensive instrumentation and reagent costs, and poor reproducibility between technicians and laboratories. Image-based cell counting is based on integrating computer software for digital image analysis with fluorescence microscopy. They are also cost-effective, easy to use, and basically maintenance-free. Another benefit is that the procedures performed by technicians are very simple and standardized, so that inter-site and inter-technician variability should be minimized [4].

We have examined the ability of the ADAM II (NanoEntek, Seoul, Korea), a newly-developed image-based cell counter, to enumerate total nucleated cells (TNCs) and viable CD34+ cells. To investigate the clinical applicability of the new equipment, we compared cell counts determined with it and those obtained with two types of flow cytometer.

Materials and Methods

1) Blood samples

We used unmanipulated 18 samples of circulating PB and peripheral blood stem cells (PBSCs) obtained from

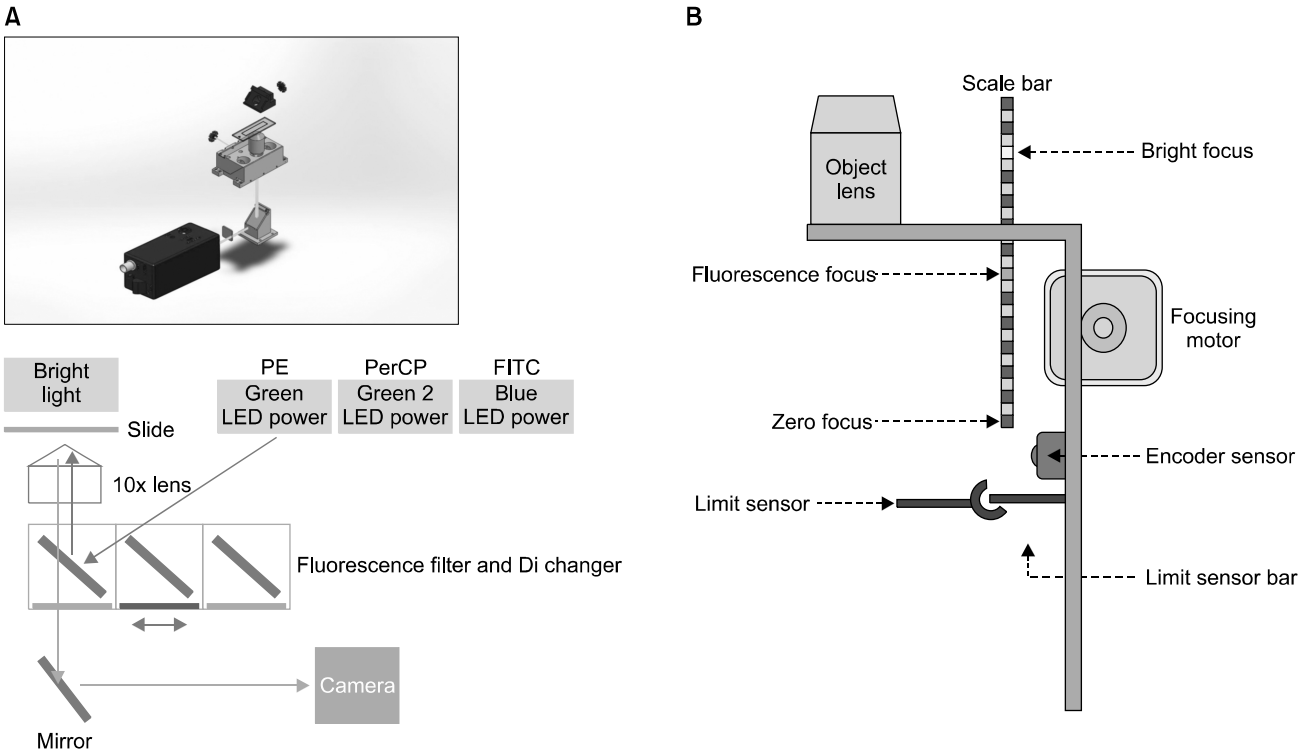

Fig. 1. (A) The ADAM II image-based fluorescence cell counter: The instrument is a 4-channel (Bright field, PE, FITC and PerCP) bench-top assay platform equipped with state-of-the-art optics and a slide holder that accepts the ADAM II assay slide. The ADAM II is based on quantitative fluorometric assay technology capable of quantifying single or multiple fluorophore(s) by measuring LED-induced fluorescence from stained cells in a single-use disposable slide. (B) The autofocusing system: the focus of the bright or fluorescence image is aligned manually in advance by turning a nob, then the z-axis movement distance (focus value) from the aligned position to the original stage position is calculated by an encoder sensor. Two or more channels (bright, fluorescence) for each image position are individually measured and set in the system. Using the established positions for each channel (bright, fluorescence), the image is focused automatically and integrated image results are obtained.

adult volunteer donors by apheresis after G-CSF mobilization. The PBSC were obtained from the tube segments that are usually discarded after cutting the apheresis tubes and retaining the harvested PBSC products. Written informed consent was obtained from all the volunteers prior to collecting samples and this study was approved by the Institutional Review Board of Hanyang University Hospital (IRB 2015-04-029).

2) Flow cytometric analysis

Two different flow cytometers, a FACSCalibur (BD Bioscience, San Jose, CA) and a FACSCanto II (BD Bioscience, San Diego, CA), in two different institutes were used. The flow cytometric analysis was performed using a BD SCE Kit (BD Bioscience, Franklin Lakes, NJ) according to the ISHAGE protocol [5]. The kit is designed to enumerate the viable dual-positive $CD45^{+dim}/CD34^{+}$ human stem cell population.

3) Image-based cell counting analysis

The image-based cell counter (ADAM II) uses a microscopic cell counting technique with disposable microchips. The system is composed of a fluorescence microscope and

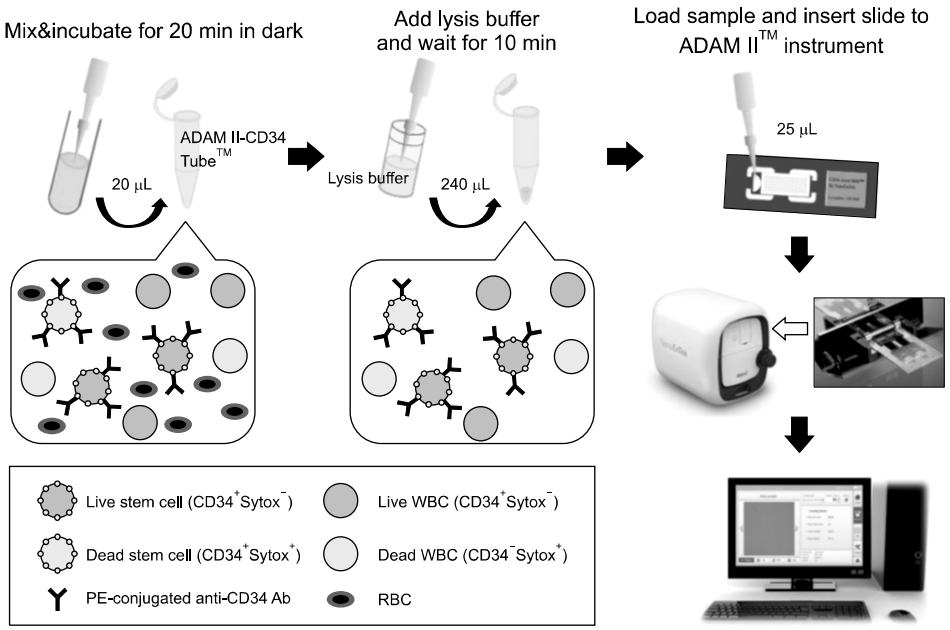

Fig. 2. The ADAM II-CD34 Counting Assay: blood samples of 20 μL are introduced into the ADAM II - CD34 tube containing lyophilized mixtures of fluorescent anti-CD34 antibody and a nucleic acid dye for cell viability. Samples are mixed and incubated for 20 minutes in the dark at room temperature (20°C to 25°C), RBC lysis buffer of 240 μL is added and after 10 minutes aliquots of 25 μL are loaded into the CD34 Assay Slide and read.

image analyzer (Fig. 1A) equipped with autofocusing function (Fig. 1B). The ADAM II is designed to be used with the ADAM II assay slide loaded with cells stained with fluorochromes (labeled antibodies against surface markers, as well as nucleic acid stains). The procedure is diagrammed in Fig. 2. The ADAM II captures simultaneously a series of bright field and fluorescence images and uses sophisticated digital image analysis algorithms to count total (TNC) and fluorescent (CD34+ cells) cells and calculate their concentrations. ADAMII takes multiple pictures (140 frames= 7.8 microliter) and pools together to generate rare cell count data such as CD34. In each frame, the system takes three pictures (bright field, FITC channel, and PE channel) by rapidly changing optic channel so that each cell can be analyzed based on its size, shape (via bright field), viability (FITC channel using Sytox blue), and cell surface marker (PE channel using CD34-PE).

4) Reproducibility and linearity of the image-based cell counter

We first examined the linearity and reproducibility of the ADAM II. Linearity of the viable CD34+ cell counts was confirmed over a range of dilutions (4-322 cells/μL of sample). Linearity was considered adequate when $R^2 > 0.98$. Reproducibility was defined as the coefficient of variation (CV) of the assay around a specific value, and was considered sufficient when CV was not in excess of 20%.

5) Statistical analysis

SPSS for Windows 16.0 (SPSS, Chicago, Illinois) was used for statistical analysis. Wilcoxon-ranksum test was used to compare the flow cytometers and ADAM II. Spearman correlation coefficients were used to assess the correlation of TNC and CD34+ cell counts between the flow cytometers and ADAM II. Statistical significance ($P < 0.05$) was determined with the nonparametric Wilcoxon test. The values of TNC and CD34+ cell counts are median values which are descripted at result part.

Table 1. Reproducibility of same sample tested 20 times

Sample number	Mean (cells/μL)	Within Stain	
		SD (cells/μL)	CV (%)
1	27.25	5.38	19.8
2	52.56	8.42	16.0
3	390.98	25.32	6.5
4	1,373.23	98.24	7.2

SD, standard deviation; CV, coefficient of variation.

Results

1) Reproducibility and linearity of the ADAM II

Each analysis used 20 aliquots from one sample, with expected values of 27-1,370 CD34+ cells/μL. The number of viable CD34+ cells determined by ADAM II was sufficiently accurate over the expected range, and the intra-assay CV was ≤19.8% (Table 1). The linearity of CD34+ cell counts was confirmed over a range of dilutions (4-322 cells/μL of sample) as shown in Fig. 3. Linearity was satisfactory (R^2=0.99).

2) Correlation between flow cytometers and ADAM II

In the samples of circulating PB, TNC counts were median of 42,193.5/μL (range, 16,032.5-68,354.5/μL) and 38,846.0/μL

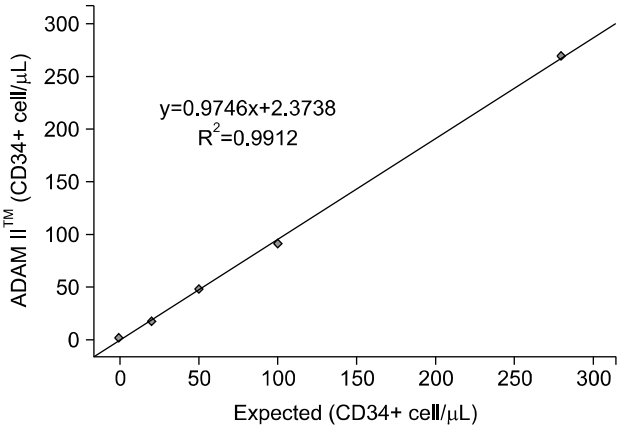

Fig. 3. The linearity between CD34+ cell counts as determined by the ADAM II and expected CD34+ cell counts in serially diluted samples. Each analysis used 5 aliquots from a dilutions (20-322 cells/μL of sample). In dilution of 20 cells/μL, mean CD34+ cell counts was 17.36±2.71, CV (%) was 15.65. In dilution of 50 cells/μL CD34+ cell count was 47.94±3.84, CV (%) was 8.00. In dilution of 100 cells/μL, CD34+ cell count was 91.34±6.28, CV (%) was 6.87. In dilution of 280 cells/μL, CD34+ cell count was 268.834±21.12, CV (%) was 7.86. In all of the dilution range (20-322 cells/μL), linearity was satisfactory (R^2=0.99).

(25,568.5-52,123.5/μL) by the 2 flow cytometers, and 45,068.5 μL (27,901.5-62,235.5/μL) and 36,373.0/μL (20,320.5-52,425.5/μL) by the ADAM II. CD34+ cell counts were 36.09/μL (0.07-72.11/μL) and 36.14/μL (15.04-57.24/μL) by the 2 flow cytometers, and 38.03/μL (9.38-85.44/μL) and 39.06/μL (16.31-61.81/μL) by the ADAM II. In the samples from apheresis, TNC counts were 242,963.5/μL (115,458.5-370,468.5/μL) and 186,825.0/μL (69,132.0-304,518.0/μL) by the 2 flow cytometers, and 208,797.5/μL (101,678.4-315,916.6/μL) and 188,329.0/μL (56,923.0-319,735.0/μL) by the ADAM II. CD34+ cell counts were 1,046.88/μL (292.73-1,798.03/μL) and 1,101.19/μL (484.13-1,718.25/μL) by the 2 flow cytometers, and 1,114.29/μL (166.62-2,081.96/μL) and 1,122.05/μL (495.75-1,748.35/μL) by the ADAM II. The comparison of TNC counts between ADAM II and FACS in PB statistically showed no difference (P=0.240). In Apheresis, TNC counts between ADAM II and FACS also showed no difference (P=0.290). The TNC and CD34+ cell counts analyzed by the flow cytometers in the 2 different institutes and by the ADAM II did not differ significantly (Fig. 4). The TNC and CD34+ cell counts obtained with the ADAM II were highly correlated with those obtained with the FACSCalibur and FACSCanto II (Fig. 5).

Discussion

Flow cytometric quantification of CD34+ cells has been widely used to optimize the mobilization and harvest of hematopoietic progenitor/stem cells for HSCT [5-7]. However, there are some disadvantages to flow cytometric analysis.

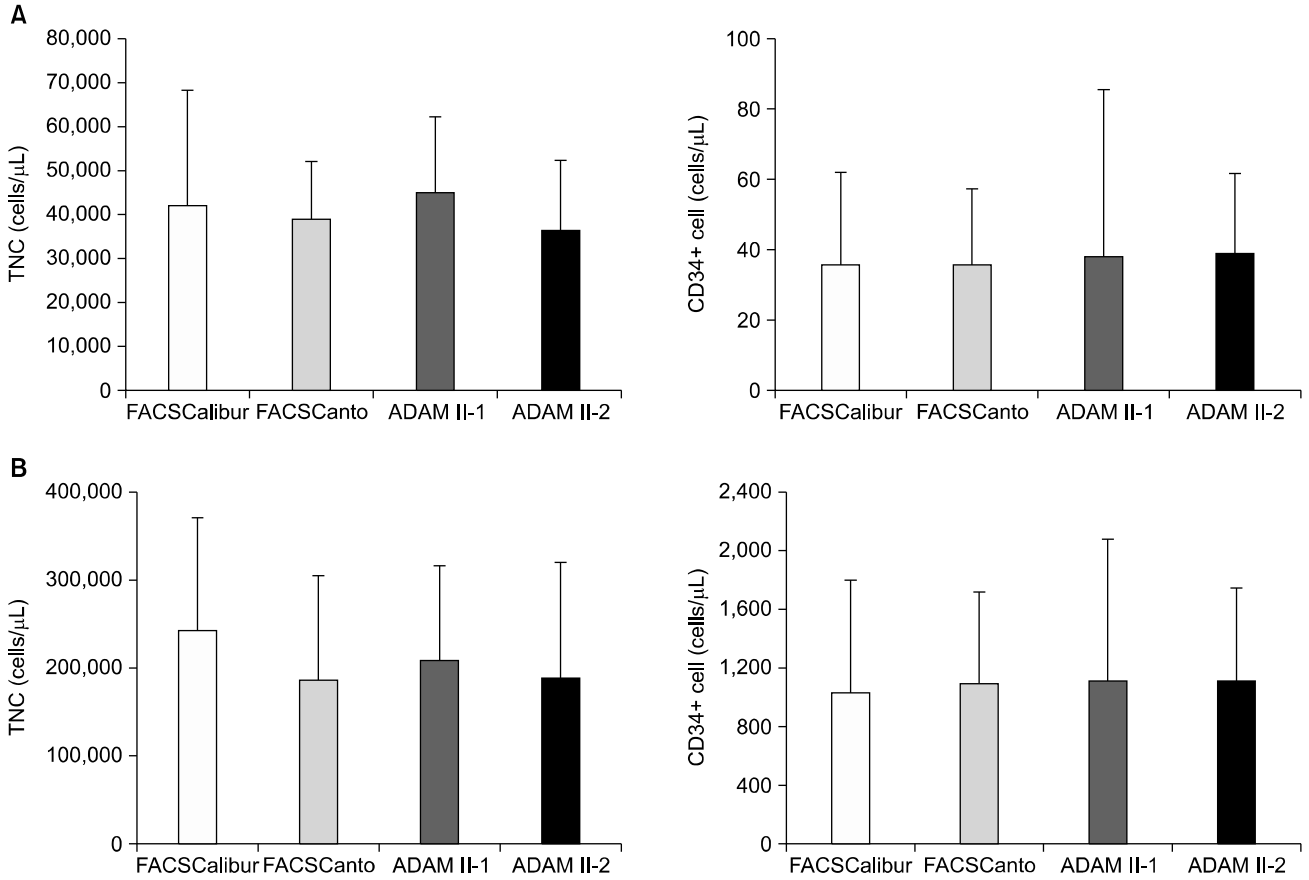

Fig. 4. (A) TNC/CD34+ cell counts in PB determined by the ADAM II in the 2 different institutes and the FACSCalibur and FACSCanto II. There was no significant differences among different equipments. Column expressed as mean of cell counts and the bar expressed as standard deviation. (B) TNC/CD34+ cell counts in apheresis samples determined by the ADAM II in the 2 different institutes and the FACSCalibur and FACSCanto II. There was no significant differences among different equipments. Column expressed as mean of cell counts and the bar expressed as standard deviation.

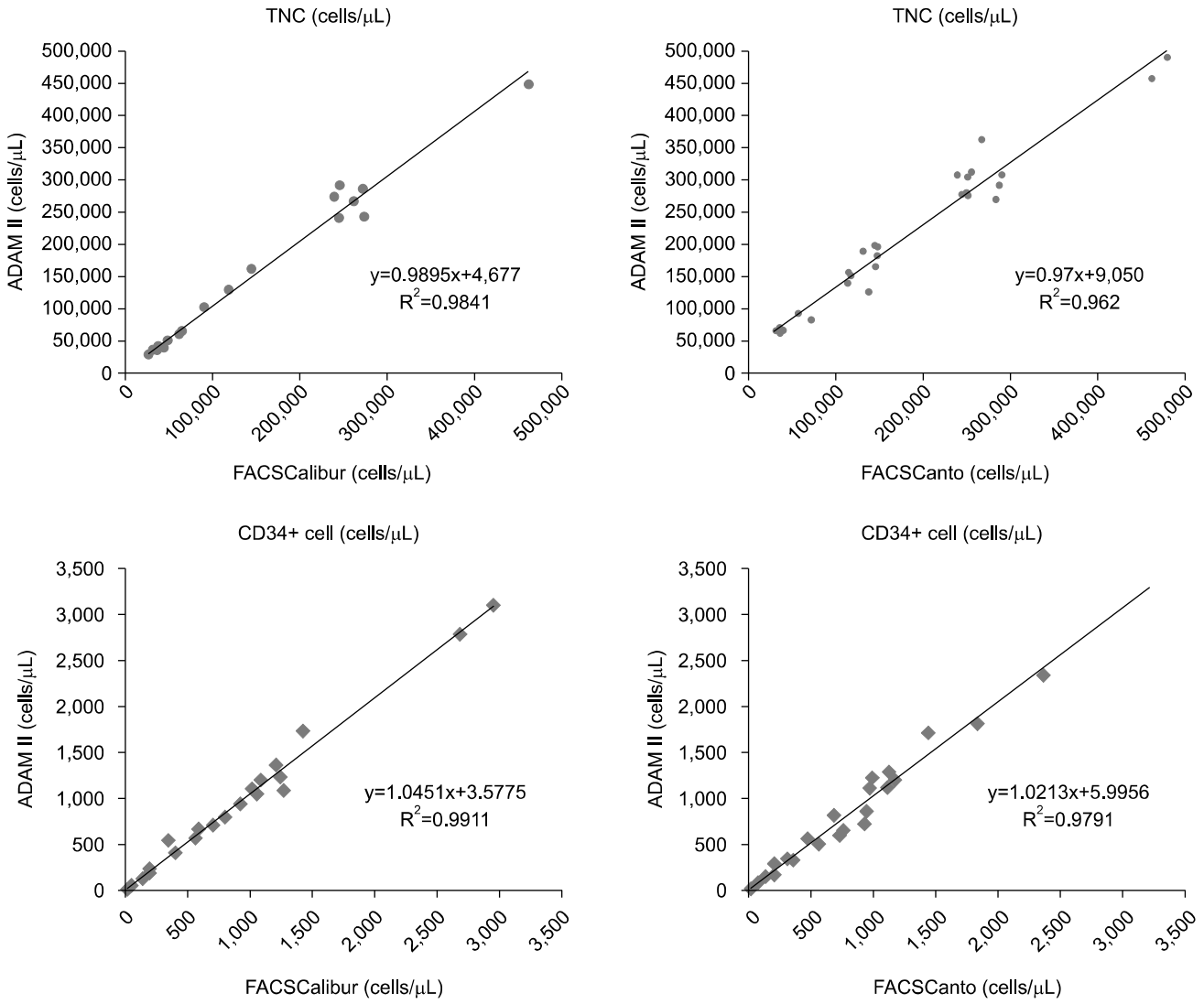

Fig. 5. Correlation between the TNC and CD34+ cell counts in the ADAM II and FACSCalibur/FACSCanto II. The TNC counts obtained with the ADAM II were highly correlated with those obtained with the FACSCalibur ($R^2>0.9841$, $P<0.0001$), and with FACSCanto II ($R^2>0.962$, $P<0.0001$), and the CD34+ cell counts obtained with the ADAM II were highly correlated with those obtained with the FACSCalibur and FACSCanto II ($R^2>0.9911$, $P<0.0001$ and $R^2>0.9791$, $P<0.0001$, respectively).

Flow cytometers, especially laser-based cell analyzers are expensive to buy and maintain. Additionally, since they are very sophisticated instruments, and only highly trained operators can run them effectively. Furthermore, flow cytometry protocols vary between institutions so that CD34+ cell counts are generally poorly reproducible between technicians and laboratories [4,8,9].

Since the late 1990s, a range of image-based automated cell counters has been launched that provide precise data on cell numbers and viability. Kummrow et al. [10] and Kim et al. [11] compared the enumeration of viable blood cells using a microscopic cell counter and flow cytometer. Although Kim found a good correlation between microscopic cell counter and flow cytometer, there is certain limitation such as difficulty in detecting unstained cells by microscope, which could lead to overestimation of the percentages of live as well as dead cells.

In order to overcome the limitations of flow cytometry and improve image-based cell counting, we have developed the ADAM II, and have used it to enumerate TNCs and CD34+ cells in this study [12]. The ADAM II is an image-based fluorescence cell counter supplied with an

all-in-one personal computer containing the software for the graphical user interface. It is based on quantitative fluorometric assay technology capable of quantifying single or multiple fluorophore(s) by measuring LED-induced fluorescence from stained cells in a single-use disposable slide. From a CD34-fluorochrome-stained and lysed sample, the ADAM II simultaneously captures a series of bright field and fluorescent images, then uses digital image analysis algorithms to determine the CD34+ cell concentrations as well as CD34+ cell viability. Each image frame covers 0.05525 μL ($850 \times 650 \times 100$ mm^3) and the counts from each frame, total 140 frames (7.8 μL) are summed to generate total counts. In our study, the minimal TNC count was 16,032.5/μL, and ADAM II counts total 7.8 μL for analyzing, so this meets the ISHAGE protocol which require at least 80,000 cells. The ADAM II also has an autofocusing system to accurately discriminate between the cells and cell debris by morphology and size. Two or more different channels (bright, fluorescence) in a given image are individually measured and set in the system. Using different position value settings for each channel (bright, fluorescence), image focusing is achieved automatically and integrated image results are obtained. In addition, Venditti et al [14] demonstrated that a single staining with anti-CD34 antibody appears feasible and reliable to enumerate CD34+ progenitor cells. These are the reason why we did not use the anti-CD45 antibody in ADAM II analysis.

In this study, we have established that cell counts determined by ADAM II are accurate, and linear with cell concentration. In addition, numbers of TNC and viable CD34+ cell counts obtained by ADAM II were highly correlated with those obtained by flow cytometry, even when the TNC counts in the ADAM II were obtained from the number of bright cells in the digital images without using anti-CD45 antibody. This study establishes the potential of the ADAM II, for providing consistent and reproducible counts of TNCs and viable CD34+ cells. Furthermore, since the data obtained with the ADAM II were highly correlated with those obtained with the current standard method for enumerating cells, we anticipate that the ADAM II could have clinical application, since the procedure is straightforward, the equipment inexpensive and there is little inter-site/-technician variability. In addition, ADAM II would be easily extended to enumerate various cell components such as CD3+, CD4+, CD8+ cells. Further studies of inter-site variability are warranted to confirm the superiority of the newly developed equipment compared to flow cytometers.

Acknowledgments

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